Amendments to the Specification

Please replace the paragraph beginning on page 1, line 18 with the following paragraph:

The Sequence Listing written in file Sequence Listing 2060_0320003.txt, 149,504 bytes, created on May 10, 2004 on two identical copies of compact discs for Application No. 09/894,018, Sette *et al.*, Method and System for Optimizing Minigenes and Peptides Encoded Thereby, is herein incorporated-by-reference.

~~The contents of the following submission on compact discs are incorporated herein by reference in its entirety: A compact disc copy of the Sequence Listing (COPY 1) (file name: 3996320033.txt, date recorded: October 25, 2001, size: 141 KB); a duplicate compact disc copy of Sequence Listing (COPY 2) (file name: 3996320033.txt, date recorded: October 25, 2001, size: 141 KB); a computer readable form copy of the Sequence Listing (CRF COPY) (file name: 3996320033.txt, date recorded: October 25, 2001, size: 141 KB).~~

Please replace the paragraph beginning on page 8, line 27 with the following paragraph:

In another embodiment, the invention provides a method and system for designing a multi-epitope construct that comprises multiple epitopes. The method comprising steps of: (i) sorting the multiple epitopes to minimize the number of junctional epitopes; (ii) introducing a flanking amino acid residue at a C+1 position of an epitope to be included within the multi-epitope construct; (iii) introducing one or more amino acid spacer residues between two epitopes of the multi-epitope construct, wherein

the spacer prevents the occurrence of a junctional epitope; and, (iv) selecting one or more multi-epitope constructs that have a minimal number of junctional epitopes, a minimal number of amino acid spacer residues, and a maximum number of flanking amino acid residues at a C+1 position relative to each epitope. In some embodiments, the spacer residues are independently selected from residues that are not known HLA Class II primary anchor residues. In particular embodiments, introducing the spacer residues prevents the occurrence of an HTL epitope. Such a spacer often comprises at least 5 amino acid residues independently selected from the group consisting of G, P, and N. In some embodiments the spacer is GPGPG (SEQ ID NO:369).

Please replace the paragraph beginning on page 17, line 26 with the following paragraph:

A "PanDR binding peptide or PADRE® ~~PADRE™~~ peptide" is a member of a family of molecules that binds more than one HLA class II DR molecule. The pattern that defines the PADRE® ~~PADRE™~~ family of molecules can be thought of as an HLA Class II supermotif. PADRE® ~~PADRE~~ binds to most HLA-DR molecules and stimulates *in vitro* and *in vivo* human helper T lymphocyte (HTL) responses.

Please replace the paragraph beginning on page 19, line 30 with the following paragraph:

The number of spacers in a construct, the number of amino acids in a spacer, and the amino acid composition of a spacer can be selected to optimize epitope processing and/or minimize junctional epitopes. It is preferred that spacers are selected by

concomitantly optimizing epitope processing and junctional motifs. Suitable amino acids for optimizing epitope processing are described herein. Also, suitable amino acid spacing for minimizing the number of junctional epitopes in a construct are described herein for class I and class II HLAs. For example, spacers flanking class II HLA epitopes preferably include G, P, and/or N residues as these are not generally known to be primary anchor residues (*see, e.g.,* PCT/US00/19774). A particularly preferred spacer for flanking a class II HLA epitope includes alternating G and P residues, for example, $(GP)_n$, $(PG)_n$, $(GP)_nG$, $(PG)_nP$, and so forth, where n is an integer between one and ten, preferably two or about two, and where a specific example of such a spacer is GPGPG (SEQ ID NO:369).

Please replace the paragraph beginning on page 26, line 1 with the following paragraph:

Figure 2 illustrates two different synthetic polypeptides (Fig. 2a) where the first construct incorporates four different epitopes linearly cosynthesized ~~eosynthetized~~, and the second construct incorporates a GPGPG (SEQ ID NO: 369) spacer. Fig. 2b illustrates the capacity of 2 nanomoles of these different constructs to prime for proliferative responses to the various epitopes in IA^b positive mice, compared to the responses induced by equimolar amounts of a pool of the same peptides (3 micrograms of each peptide).

Please replace the paragraph beginning on page 27, line 6 with the following paragraph:

Figure 9 shows HIV multi-epitope constructs optimized using the methods of the present invention. GAAA spacer (SEQ ID NO:380); NAAA spacer (SEQ ID NO:381); and KAAA spacer (SEQ ID NO:382).

Please replace the paragraph beginning on page 27, line 21 with the following paragraph:

Figure 15 shows the effect of GPGPG (SEQ ID NO: 369) spacers in class II epitope constructs HIV 75mer and HIV 60mer on HTL responses to particular epitopes.

Please replace the paragraph beginning on page 27, line 23 with the following paragraph:

Figure 16 depicts HTL responses to particular epitopes present in the EP-HIV-1043-PADRE® ~~EP-HIV-1043-PADRE~~ construct.

Please replace the paragraph beginning on page 27, line 25 with the following paragraph:

Figure 17 is a schematic depicting the epitopes present in HIV 75mer, EP-HIV-1043, and EP-HIV-1043-PADRE® ~~EP-HIV-1043-PADRE~~.

Please replace the paragraph beginning on page 32, line 7 with the following paragraph:

In one embodiment, to correct the problem of junctional epitopes for HTL epitopes, a spacer of, for example, five amino acids in length is inserted between the two

epitopes. The amino acid residues incorporated into such a spacer are preferably those amino acid residues that are not known to be primary anchor residues for any of the HLA Class II binding motifs. Such residues include G, P, and N. In a preferred embodiment, a spacer with the sequence GPGPG (SEQ ID NO: 369) is inserted between two epitopes. Previous work has demonstrated that the GP spacer is particularly effective in disrupting Class II binding interactions (Sette et al., *J. Immunol.*, 143:1268-73 (1989)). All known human Class II binding motifs and the mouse IA^b (the Class II expressed by HLA transgenic mice) do not tolerate either G or P at this main anchor positions, which are spaced four residues apart. This approach virtually guarantees that no Class II restricted epitopes can be formed as junctional epitopes.

Please replace the paragraph beginning on page 33, line 3 with the following paragraph:

The first construct incorporates four different epitopes linearly arranged, while the second construct incorporates the GPGPG (SEQ ID NO: 369) spacer. Synthetic peptides corresponding to the three potential junctional epitopes were also synthesized.

Please replace the paragraph beginning on page 33, line 6 with the following paragraph:

The capacity of 2 nanomoles of these different constructs to prime for proliferative responses to the various epitopes in IA^b positive mice was tested, and compared to the responses induced by equimolar amounts of a pool of the same peptides (3 micrograms of each peptide). Specifically, groups of 3 mice were injected with

peptides in CFA emulsions, 11 days after injection their lymph node cells were cultured *in vitro* for an additional 3 days, and thymidine incorporation was measured in the last 24 hours of culture. It was found (Fig. 2b) that, as predicted on the basis of their high affinity IA^b binding capacity, all four epitopes induced good proliferation responses. Stimulation index (SI) values in the 4.9 to 17.9 range were observed when these peptides were injected in a pool. However, when the linear polypeptide incorporating the same epitopes was tested, the response directed against Pol 335 was lost. This was associated with appearance of a response directed against a junctional epitope spanning Gag 171 and Pol 335. The use of the GPGPG (SEQ ID NO:369) spacer eliminated this problem, presumably by destroying the junctional epitope, and the Pol 335 response was regained. The responses observed were of magnitude similar to those observed with the pool of isolated peptides.

Please replace the paragraph beginning on page 34, line 19 with the following paragraph:

Table 3. Differences in effectiveness of T cell priming for specific epitopes in different minigenes.

Epitope Identity	Minigene Identity	Flanking Sequence (N terminus)	Epitope Sequence ~~(Seq.Id.Nos:1-4)~~	Flanking Sequence (C terminus)	(SEQ ID NO:)	Immune Response Frequency	Immune Response Magnitude [1]
Core 18	HBV.1	TLKAAA	FLPSDFFPSV	FLLSLG	1	6/6	5.5
	pMin1	TLKAAA	FLPSDFFPSV	KLTPLC	2	6/6	1074.5
Core 132	HCV1	ILGGWV	DLMGYIPLV	YLVAYQ	3	2/12	107.7
	HCV2	VPGSRG	DLMGYIPLV	AKFVA	4	17/18	929.2

[1] IFNγ secretory units

Please replace the paragraph beginning on page 42, line 25 with the following paragraph:

The motifs 206 in the text file 200 provide a "mask" or structural model for identifying junctional epitopes. For example the first motif 206a shown in Figure 11, XXXX(FY)XX(LIMV) (SEQ ID NO: 370) ~~(SEQ ID NOS: 7-14)~~, defines an epitope that is eight amino acids in length. The value "X" indicates that any amino acid may be at that position of the epitope. The value "(FY)" indicates that either an F amino acid or a Y amino acid may be in the fifth position of the epitope. Similarly, "(LIMV)" indicates that any one of the listed amino acids, L, I, M or V, may be in the eighth position of the epitope. Therefore if a sequence of eight amino acids spanning a junction of two peptides satisfies the above motif criteria, it is identified as a junctional epitope.

Please replace the paragraph beginning on page 56, line 8 with the following paragraph:

Multiple HLA class II or class I epitopes present in a multi-epitope construct can be derived from the same antigen, or from different antigens. For example, a multi-epitope construct can contain one or more HLA epitopes that can be derived from two different antigens of the same virus or from two different antigens of different viruses. Epitopes for inclusion in a multi-epitope construct can be selected by one of skill in the art, *e.g.*, by using a computer to select epitopes that contain HLA allele-specific motifs or supermotifs. The multi-epitope constructs of the invention can also encode one or more broadly cross-reactive binding, or universal, HLA class II epitopes, *e.g.*, PADRE®

~~PADRE™~~ (Epimmune, San Diego, CA), (described, for example, in U.S. Patent Number 5,736,142) or a PADRE® ~~PADRE™~~ family molecule.

Please replace the paragraph beginning on page 71, line 6 with the following paragraph:

This example provides an example of testing multiple ~~mutliple~~ CTL and HTL epitopes. For example, epitope strings encompassing 10-12 different CTL epitopes under the control of a single promoter are synthesized and incorporated in a standard plasmid, pcDNA 3.1 (Invitrogen, San Diego). These constructs include a standard signal sequence and a universal HTL epitope, PADRE® ~~PADRE™~~. Each set of epitopes is chosen to allow balanced population coverage. To facilitate testing and optimization, a balanced representation of epitopes that have been shown to be immunogenic in transgenic mice, and/or antigenic in humans are included.

Please replace the paragraph beginning on page 73, line 6 with the following paragraph:

Epitope strings encompassing 3-20 different HTL epitopes under the control of a single promoter are synthesized and incorporated into a standard plasmid, pcDNA 3.1 (Invitrogen, San Diego). To facilitate testing and optimization, each set of epitopes for a given minigene is chosen to provide a balanced representation of epitopes which are already known to be immunogenic in IA^b mice. In addition, all the peptides corresponding to junctions are synthesized and tested for binding IA^b and, most importantly, for binding to a panel of fourteen different DR molecules, representative of

the most common DR alleles worldwide (Southwood et al., *J Immunol*, Vol. 160(7):3363-73 (1998)). Thus, HTL epitopes that are not directed to an antigen of interest are not created within these plasmids. However, should junctional regions with good DR binding potential (and hence potential DR restricted immunogenicity *in vivo*) be detected, a spacer such as GPGPG (SEQ ID NO: 369) is introduced to eliminate them. In all constructs, the number of Class I junctional motifs will also be minimized, as described herein.

Please replace the paragraph beginning on page 74, line 1 with the following paragraph:

These minigene plasmids are also used to determine the effects of HTL epitopes on responses to CTL epitopes. Specifically, HTL and CTL containing plasmids are pooled and injected in mice, and CTL and HTL responses to selected epitopes are measured as described herein. Often, it is determined whether the presence, *e.g.*, of HTL epitopes derived from the target antigen enhances CTL responses beyond the level of response attained using a plasmid-containing a pan DR binding epitope, *e.g.*, PADRE® ~~PADRE™~~ or a PADRE® ~~PADRE~~ family molecule, in the CTL minigene. Typically, it is also determined whether PADRE® ~~PADRE~~ inhibits or augments responses to target antigen-derived HTL epitopes or conversely, if HTL epitopes derived from the antigen of interest inhibit or augment responses to PADRE® ~~PADRE~~.

Please replace the paragraph beginning on page 75, line 18 with the following paragraph:

For HTL minigene vaccines, the data obtained from the "first generation" minigene vaccines are inspected for trends, in terms of junctional epitopes, and epitope position within the minigene, and proximity to spacers, *e.g.* GPGPG (SEQ ID NO: 369) spacers. If specific trends are detected, second generation minigene vaccines are designed based on these trends. Alternatively, in case of minigenes yielding suboptimal activity, the potential effectiveness of other targeting strategies, such as the ones based on Ii and LAMP are reevaluated, and compared to no targeting and simple, leader sequence targeting.

Please replace the paragraph beginning on page 78, line 6 with the following paragraph:

Optimized constructs were designed with the aid of the computer assisted methods described above which simultaneously minimize the formation of junctional epitopes and optimize C+1 processing efficiency. The following motifs were utilized for junctional minimization: murine K^b [XXXX(FY)X_{2-3}(LIMV)] (SEQ ID NOS: 370 and 371) ~~(SEQ ID NOS: 7-22)~~; D^b [XXXXNX$_{2-3}$LIMV)] (SEQ ID NOS:372 and 373) ~~(SEQ ID NOS: 23-30)~~; human A2 [X(LM)X_{6-7}V] (SEQ ID NOS: 374 and 375) ~~(SEQ ID NOS: 31-34)~~; human A3/A11 [X(LIMV)X_{6-7}(KRY)] (SEQ ID NOS: 376 and 377) ~~(SEQ ID NOS:35-38)~~; and human B7 [XPX_{6-7}(LIMVF)] (SEQ ID NOS: 378 and 379) ~~(SEQ ID NOS:59-68)~~. The C+1 propensity values were calculated from the data presented in Figure 6 and are as follows: K = 2.2; N = 2; G = 1.8; T = 1.5; A,F,S = 1.33; W,Q = 1.2; R = 1.7; M,Y = 1; I = 0.86; L = 0.76; V,D,H,E,P = 0. Insertion of up to four amino acids was permitted. Examples of constructs designed by this procedure and other procedures

set forth herein are depicted in Figure 19. A number of these constructs were characterized *in vitro* and *in vivo* immunogenicity studies, which are set forth hereafter. Figure 20 lists amino acid epitope sequences encoded by certain nucleic acid sequence set forth in multiepitopic constructs.

Please replace the paragraph beginning on page 79, line 16 with the following paragraph:

A universal spacer consisting of GPGPG (SEQ ID NO: 369) was developed to separate HTL epitopes, thus disrupting junctional epitopes. The logic behind the design of this spacer is that neither G nor P are used as primary anchors, positions 1 and 6 in the core region of an HTL peptide epitope, by any known murine or human MHC Class I or MHC ~~IMHC~~ Class II molecule. The gap of five amino acids introduced by this spacer separates adjacent epitopes so the amino acids of two epitopes cannot physically serve as anchors in the 1 and 6 positions. The utility of the GPGPG (SEQ ID NO: 369) spacer was tested using synthetic peptides composed of four HIV-1 epitopes, one having three spacers and the other lacking spacers, known to bind mouse IA^b. HIV 75mer was the construct having three GPGPG (SEQ ID NO: 369) spacers and HIV 60mer was the construct lacking the three spacers. Immunization of CB6F1 mice with the peptide in CFA induced HTL responses against 3 of 4 of the epitopes in the absence of the spacer but all epitopes were immunogenic when the spacer was present (Figure 15). This evidence demonstrates that spacers can improve the performance of multi-epitope constructs.

Please replace the paragraph beginning on page 79, line 29 with the following paragraph:

The ability of multi-epitope HTL DNA-based constructs to induce an HTL response *in vivo* was evaluated by intramuscular immunization of $H2^{bxd}$ mice with an HIV-1043-PADRE® ~~HIV-1043-PADRE~~ construct. The HIV-1043-PADRE® ~~HIV-1043-PADRE~~ construct is set forth in Figure 19, and the difference between HIV-1043-PADRE® ~~HIV-1043-PADRE~~ and HIV-1043 is that the former includes a C-terminal GPGPG (SEQ ID NO:369) spacer followed by the PADRE® ~~PADRE~~ sequence AKFVAAWTLKAAA (SEQ ID NO:69). Eleven days after immunization, no booster immunizations were administered, CD4 T cells were purified from the spleen, and peptide specific HTL responses were measured in a primary γ-IFN ELISPOT assay. Examples of HTL activity induced by constructs encoding HIV epitopes are shown in Figure 17. Overall, the HTL responses induced by DNA immunization with the multiepitope HIV HTL construct were generally of equal or greater magnitude than the responses induced by peptide immunization.